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Original Article

Self-reported sleep duration, white blood cell counts and cytokine profiles in European adolescents: the HELENA study

Fátima Pérez de Heredia [a,b,*,†], Marta Garaulet [c,†], Sonia Gómez-Martínez [a], Ligia E. Díaz [a], Julia Wärnberg [d], Odysseas Androutsos [e], Nathalie Michels [f], Christina Breidenassel [g,h], Magdalena Cuenca-García [i], Inge Huybrechts [j], Frédéric Gottrand [k], Marika Ferrari [l], Alba M. Santaliestra-Pasías [m], Anthony Kafatos [n], Denes Molnár [o], Michael Sjöstrom [d], Kurt Widhalm [p], Luis A. Moreno [m,q], Ascensión Marcos [a], on behalf of the HELENA Study Group [1]

[a] Immunonutrition Research Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain
[b] School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool, United Kingdom
[c] Department of Physiology, Faculty of Biology, University of Murcia, Murcia, Spain
[d] Unit for Preventive Nutrition, Department of Biosciences and Nutrition at Novum, Karolinska Institutet, Stockholm, Sweden
[e] Department of Nutrition and Dietetics, University of Harokopio, Athens, Greece
[f] Department of Public Health, Faculty of Medicine and Health Sciences, University of Ghent, UZ-Ghent, Gent, Belgium
[g] Department of Nutrition and Food Science, University of Bonn, Bonn, Germany
[h] ImFINE Research Group, Department of Health and Human Performance, Faculty of Physical Activity and Sports Sciences-INEF, Technical University of Madrid, Madrid, Spain
[i] Department of Medical Physiology, School of Medicine, University of Granada, Granada, Spain
[j] Dietary Exposure Assessment Group, International Agency for Research on Cancer, Lyon, France
[k] Hôpital J de Flandre CHRU de Lille, Inserm U995, Faculté de Médecine, Université de Lille 2, Lille, France
[l] National Research Institute on Food and Nutrition, Rome, Italy
[m] GENUD (Growth, Exercise, Nutrition and Development) Research Group, University of Zaragoza, Zaragoza, Spain
[n] Preventive Medicine and Nutrition Unit, School of Medicine, University of Crete, Heraklion, Greece
[o] Department of Pediatrics, University of Pécs, Pécs, Hungary
[p] Department of Pediatrics, Division of Clinical Nutrition and Prevention, Medical University of Vienna, Vienna, Austria
[q] Department of Preventive Medicine, University of São Paulo, São Paulo, Brazil

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ABSTRACT

Background: Sleep patterns face important changes during adolescence. This can have implications for the immune system, which is regulated by the sleep–wake cycle; however, most studies relating sleep and immune system have been conducted on adults.
Objective: To study the relationships between sleep duration, immune cell counts, and cytokines in European adolescents participating in the HELENA Cross-Sectional Study.
Methods: Adolescents (12.5–17.5 years; $n = 933$; 53.9% girls) were grouped according to self-reported sleep duration into <8, 8–8.9 and ≥9 h/night. Blood samples were collected in the morning after an overnight fast to analyze counts of white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, the lymphocyte subsets $CD3^+$, $CD4^+$, $CD8^+$, $CD45RA^+$, $CD45RO^+$, $CD3^-CD16^+56^+$ and $CD19^+$, and concentrations of cortisol, CRP, IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α and IFN-γ. Pro-/anti-inflammatory and Th1/Th2 cytokine ratios were calculated. Immune parameters were correlated to sleep duration and compared between the three groups.
Results: Sleep duration was negatively associated with cortisol levels and WBC, neutrophil, monocyte, $CD4^+$ and $CD4^+CD45RO^+$ counts; in girls it is also negatively associated with IL-5 and IL-6 levels. The 8–8.9 h/night group presented the highest IL-4 values and the lowest pro-/anti-inflammatory and Th1/Th2 cytokine ratios.
Conclusion: A sleep duration of 8–8.9 h/night was associated with a healthier immune profile in our adolescents.

* Corresponding author at: School of Natural Sciences and Psychology, Liverpool John Moores University, James Parsons Bld, Byrom St, Liverpool L3 3AF, United Kingdom. Tel.: +44 (0) 1512 312003; fax: +44 (0) 151 2312338.
E-mail address: f.perezdeherediabenedicte@ljmu.ac.uk (F. Pérez de Heredia).
† These authors contributed equally to this article.
[1] Healthy Lifestyle in Europe by Nutrition in Adolescence – see Appendix.

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1. Introduction

Most functions within the human body present a rhythmicity of ~24 h (circadian rhythms); the circulating levels of our hormones, cytokines and metabolites experience regular daily variations that are controlled by a central clock [1]. Environmental and behavioral changes such as alternation between light and darkness, intake and fasting, or activity and repose are key regulators of this central clock. The breakdown of the normal phase relationship between the internal circadian rhythms and the 24 h environmental cycles is named chronodisruption. In our modern society, chronodisruption can be the result of several conditions such as jet lag, shift working, or night eating [2]. Among adolescents, the main agent of chronodisruption is lack of sleep. Sleep patterns are often altered in this age period; however, adequate sleep is a critical factor for adolescents' health and the establishment of health-related behaviors [3].

In recent years, sleep duration has decreased significantly in the adult population, a situation that has been related to various metabolic disorders [4]. Lack of sleep is associated with higher risk of suffering from infections and worse recovery, and with increased risk of developing non-infectious diseases and chronic low-grade inflammation [5]. These conditions are linked to alterations in the immune response, which may be secondary to the activation of the stress axis and the release of cortisol, a well-known regulator of immune activity; however, it cannot be ruled out that changes in immune parameters respond to a direct cross-talk between sleep and the immune system [6–8].

The immune system shows circadian rhythms in its cell counts and in its functions [9]. In addition, peripheral circadian clocks have been found in various white blood cell (WBC) types [7,10,11]. Different experiments have been conducted in the laboratory setting to study the effects of sleep on immune function. The majority explore the effects of short-term (acute) lack of sleep, and adopt one of two strategies: controlled sleep restriction, in which sleep duration is limited to a few hours (frequently five or less), and controlled sleep deprivation, in which subjects experience continuous wakefulness for a certain period of time (generally 24–48 h). These experiments have shown that lack of sleep is followed by elevations in WBC counts, notably in neutrophils and/or monocytes [12–15]. Sleep is believed to stimulate the specific (adaptive) immune response, whereas wakefulness seems to favor non-specific responses (innate immunity) mediated mainly by neutrophils, monocytes and natural killer (NK) cells (Table 1) [5,7]. The expression and production of cytokines from immune cells follow a circadian pattern that can be synchronized with sleep and wakefulness [9]. Therefore, the sleep–wake cycle may influence immune function.

There is a limitation, however, to the interpretation of the information available on the relationship between sleep (especially lack of sleep) and immune function. While sleep restriction and sleep deprivation studies provide very useful information on the effects of both acute and more prolonged lack of sleep, we cannot be certain that, in a real life-setting, less dramatic but habitual lack of sleep will trigger the same response in the body. In other words, adaptive responses may differ with time, and so may their impact on the individual's health.

The vast majority of studies have been conducted on adults and little information is available on adolescents. However, as mentioned above, adolescence is a period of great interest, due to the many physiological and behavioral changes taking place; these include changes in sleep habits, characterized mainly by delayed sleep onset leading to shortening of sleep duration. For these reasons, we aimed to study whether sleep duration is related to immune parameters in a representative sample of European adolescents.

2. Methods

2.1. Study design and sample selection

A European multicenter cross-sectional study (CSS) was performed with the objective to assess a "healthy lifestyle in Europe by nutrition in adolescence" (HELENA). The HELENA-CSS aimed to obtain reliable and comparable data on nutrition and other health indicators such as physical activity and fitness, body composition, cardiovascular disease risk factors, vitamin and mineral status, and immunological and genetic markers in European adolescents [16]. The methodology used in this study has been published elsewhere [17]. The study was performed according to the ethical guidelines of the Edinburgh revision of the 1964 Declaration of Helsinki (2000), the International Conferences on Harmonization for Good Clinical Practice and the legislation on clinical research from each of the participating countries. The protocol was approved by the Research Ethics Committees of each center involved. Written informed consent was obtained from the adolescents and their parents [18].

Briefly, subjects aged 12.5–17.5 years were recruited from 10 cities belonging to nine countries across Europe (Athens and Heraklion in Greece, Dortmund in Germany, Ghent in Belgium, Lille in France, Pècs in Hungary, Rome in Italy, Stockholm in Sweden, Vienna in Austria, and Zaragoza in Spain). The total eligible HELENA-CSS population consisted of 3528 adolescents, with inclusion criteria being not participating simultaneously in another clinical trial and being free of any acute infection occurring within the week prior to the study.

2.2. Sleep hours determination

Habitual sleep time was estimated by a questionnaire. The adolescents were asked the following questions: "How many hours (and minutes) do you usually sleep during week days?" and "How many hours (and minutes) do you usually sleep during weekend days?" A total weekly sleep score was calculated as: [(min weekday × 5) + (min weekend day × 2)]/7.

Table 1
Summary of the different white blood cell (WBC) types analyzed.

WBC types	Lymphocyte subsets	Membrane markers	Immune function
Neutrophils			Innate or non-specific
Monocytes			Innate or non-specific
Lymphocytes	Mature T-cells	$CD3^+$	Adaptive or specific
	Naive T-helper	$CD3^+CD4^+CD45RA^+$	Adaptive or specific
	Memory T-helper	$CD3^+CD4^+CD45RO^+$	Adaptive or specific
	Naive T-cytotoxic	$CD3^+CD8^+CD45RA^+$	Adaptive or specific
	Memory T-cytotoxic	$CD3^+CD8^+CD45RO^+$	Adaptive or specific
	B-cells	$CD3^-CD19^+$	Adaptive or specific
	Natural killer cells	$CD3^-CD16^+56^+$	Innate or non-specific
Eosinophils			Innate or non-specific
Basophils			Innate or non-specific

The reliability of the sleep report was studied in 183 adolescents (aged 13–18 years), who completed a questionnaire twice, 1 week apart. This subsample did not participate in the HELENA Study, yet it did not differ in age, ethnicity or socio-economic status from the final HELENA-CSS sample. Questions used to estimate sleep time were reliable; Cohen's weighted kappa showed an almost perfect agreement (0.81 and 0.96 for weekdays and weekend days, respectively).

The total sleep score (the weighted mean of sleep during weekdays and weekends) was used to classify our population into three groups according to sleep duration: < 8, 8–8.99, and ≥9 h/night. According to the criterion of the National Sleep Foundation for the adolescent population, < 8 h/night is defined as insufficient sleep [19], and experimental evidence has suggested that 9 h/night is the optimal sleep duration at this age period [20].

2.3. Blood sampling and measurements of immune parameters

Blood samples were obtained from one-third of the participants, resulting in a subpopulation of 1089 adolescents (~100 males and females per center); the size of this subpopulation was previously calculated as sufficient to account for the expected variability in blood measurements. Venous blood samples were collected in EDTA Monovette (Sarstedt, Germany) tubes between 08:00 a.m. and 10:00 a.m. after a 12 h overnight fast. WBC counts were determined in each participating city with automated blood cell counters. The methodology for blood collection, transport and analysis was standardized among all participating centers [21].

For lymphocyte subset analysis, blood aliquots were taken into 1.5 mL plastic tubes, diluted 1:1 with Cytochex™ Reagent (Streck Laboratories, Omaha, NE, USA), and sent to the Immunonutrition laboratory at the Spanish National Research Council (CSIC) in Madrid, where they were analyzed within seven days from collection. Briefly, blood aliquots were incubated with monoclonal antibodies (BD Biosciences, San José, CA, USA) for 30 min at room temperature, to label the following cells differentially (see also Table 1): $CD45^+$ (the pan-leukocyte marker), $CD3^+$ (mature T-cells), $CD4^+$ (T-helper cells), $CD8^+$ (T-cytotoxic cells), $CD19^+$ (B-cells), $CD3^-CD16^+56^+$ [natural killer (NK) cells], $CD45RO^+$ (memory cells) and $CD45RA^+$ (naive cells). After a lyse/wash procedure with a fixation step using BD FACS lysis solution (BD Biosciences), samples were analyzed by flow cytometry (FACScan Plus Dual Laser, BD, Sunnyvale, CA, USA). Lymphocytes were gated by forward and side scatter and CD45 expression, and by a simultaneous four-staining procedure: CD45RA/CD45RO/CD8/CD3, CD45RA/CD45RO/CD4/CD3, CD3/$CD16^+$56/CD45/CD19, and CD3/CD8/CD45/CD4. Percentages of lymphocyte subsets were measured relative to the main corresponding population, and absolute cell counts were estimated in relation to total lymphocyte counts.

For analysis of serum proteins, blood was collected in Vacutainer™ tubes (BD Biosciences) and allowed to clot. Within the hour, serum was separated by centrifugation at 3500 rpm for 15 min; aliquots were made and sent to Bonn in cooled containers on the same day, and stored in Bonn at –80 °C. At the end of the study, all samples were sent to Madrid (CSIC) on dry ice and stored at –80 °C until analysis. Serum levels of interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-10, tumor necrosis factor-α (TNF-α), and γ-interferon (IFN-γ) were measured using the High Sensitivity Human Cytokine Milliplex™ MAP kit (MPXHCYTO-60K) (Millipore Corp., Billerica, MA, USA) and collected by flow cytometry (Luminex-100 v.2.3, Luminex Corporation, Austin, TX, USA). C-reactive protein (CRP) levels were quantified by immunoturbidimetry (AU 2700, Olimpus, Rungis, France). For cortisol measurement, saliva samples were taken and analyzed as described elsewhere [22]. Briefly, saliva was collected with Salivettes® (Sarstedt, Germany) and cortisol was measured using a modification of an unextracted radioimmunoassay method (Diasorin) for serum cortisol.

From the immune and cytokine measurements, different ratios were calculated: (i) T-helper/T-cytotoxic cell ratio ($CD4^+/CD8^+$ cell counts); (ii) naive/memory cell ratios ($CD3^+RA^+/CD3^+RO^+$, $CD4^+RA^+/CD4^+RO^+$ and $CD8^+RA^+/CD8^+RO^+$ cell counts); (iii) pro-inflammatory/anti-inflammatory cytokine ratio [calculated as (TNF-α + IL-1)/(IL-4 + IL10)]; and (iv) Th1/Th2 cytokine ratio [calculated as (TNF-α + IFN-γ + IL-2)/(IL-4 + IL5 + IL-6)].

2.4. Anthropometric measurements

To account for the confounding effect of physiological development on immune function, identification of the degree of pubertal maturation was assessed by a medical doctor, according to the Tanner and Whitehouse classification [23].

Standardized body mass index (BMI) (*z*-scores) was considered when analyzing data, as it was previously found to be significantly associated with sleep duration in this adolescent population [24]. Anthropometric procedures were standardized in all participating centers and have been described elsewhere [25]. Briefly, weight was measured in underwear and without shoes using an electronic scale (Type SECA 861) to the nearest 0.1 kg, and height was measured barefoot in the Frankfort plane with a telescopic height-measuring instrument (Type SECA 225) to the nearest 0.1 cm. BMI was then calculated as: body weight (kg)/height (m^2). Gender- and age-standardized BMI values (*z*-scores) were used for subsequent statistical analyses.

2.5. Statistical methods

All the analyses conducted on the HELENA-CSS data were adjusted by a weighing factor to balance the studied population according to the age and gender distribution of the theoretical sample. Those adolescents who did not provide data on their sleep duration or a valid Tanner stage were excluded from the analysis, giving a final sample of 933 adolescents (53.9% girls).

Normality of immune variables was checked by the Kolmogorov–Smirnov test, and those not normally distributed were appropriately log- or square-root-transformed. The general characteristics of the three sleep duration groups (<8, 8–8.99 and ≥9 h/night) were compared by means of one-way analysis of variance (ANOVA) for continuous variables (average sleep duration, sleep hours during weekdays and sleep hours during the weekend), and by the χ^2-test for categorical variables (center, gender, and Tanner stage).

Partial correlations between sleep duration and all immune parameters studied (white blood cells, lymphocyte subsets, cortisol, and serum proteins) were analyzed controlling for the following confounding factors: gender, data collection site (here named "center"), Tanner stage, and BMI. Values of immune parameters were compared between the three sleep groups by one-way analysis of covariance (ANCOVA), adjusting for gender, center, Tanner stage, and BMI. The center was included as a covariate to account for potential variability due to geographical, genetic, and cultural differences among countries and cities. Tanner stage was chosen as covariate instead of age (years) of the adolescents, since physiological age can be more accurate than chronological age at this developmental period. To confirm the suitability of the choice, analyses were also made adjusting for age instead of Tanner stage, yielding similar results (data not shown).

Statistical significance was set at $P < 0.05$. All statistical analyses were performed with IBM SPSS Statistics v.19 for Windows.

Table 2
Characteristics of the adolescent population studied, classified according to sleep duration.

	All	Sleep categories		
		<8 h/night	8–8.99 h/night	≥9 h/night
n (%)	933	291 (31.2)	388 (41.6)	254 (27.2)
Boys	430 (46.1)	125 (29.1)*	172 (40.0)*	134 (31.2)*
Girls	503 (53.9)	166 (33.0)*	216 (42.9)*	120 (23.9)*
Age (mean ± SD)	14.9 ± 1.2	15.4 ± 1.1*	14.9 ± 1.3*	14.5 ± 1.2*
Tanner stage (%)				
I	0.6	0*	0.8*	1.2*
II	5.6	2.1*	6.4*	8.6*
III	18.9	15.4*	19.5*	22.0*
IV	43.6	46.9*	41.4*	42.7*
V	31.3	35.6*	31.9*	25.5*
Average sleep duration (h/night)	8.09 ± 1.15	6.85 ± 0.64*	8.12 ± 0.22*	9.47 ± 0.78*
On weekdays	8.05 ± 1.12	6.82 ± 0.67*	8.12 ± 0.30*	9.37 ± 0.67*
On weekends	8.17 ± 1.42	6.90 ± 0.85*	8.11 ± 0.45*	9.74 ± 1.40*

* Significant difference ($P < 0.05$) between sleep duration groups, as assessed by one-way analysis of variance (for age and sleep duration), or by Pearson's χ^2-test (for percentages of gender and Tanner stages).

3. Results

3.1. Characteristics of the population

Table 2 summarizes the characteristics of the population studied. Average daily sleep duration in the total sample was 8.1 ± 1.2 h (range: 5–13.5 h/night). There were significant differences in the frequency of sleep categories between genders ($\chi^2 = 6.17$, $P = 0.046$). The percentage of adolescents sleeping <8 h/night was higher in girls than in boys (33% vs 29%, respectively), and the same was observed for the percentages of adolescents sleeping 8–8.99 h/night (43% in girls vs 40% in boys); conversely, the percentage of adolescents sleeping ≥9 h/night was higher in boys than in girls (31% vs 24%).

3.2. White blood cell counts and indices in relation to sleep duration

In relation to cell counts, significant negative correlations were found between sleep duration and total WBC ($r = –0.082$, $P = 0.013$), neutrophils ($r = –0.069$, $P = 0.048$), monocytes ($r = –0.066$, $P = 0.047$), and memory T-helper cells ($CD4^+$) ($r = –0.121$, $P = 0.001$), all showing higher cell counts with shorter sleep durations.

With regard to lymphocyte subsets, adolescents in the <8 h/night group had higher counts of total T-cells ($CD3^+$; $P = 0.040$), total memory cells ($CD3^+RO^+$; $P = 0.024$), T-helper cells ($CD4^+$; $P = 0.006$), and T-helper memory cells ($CD4^+RO^+$; $P < 0.001$) compared with the 8–8.99 h/night and the ≥9 h/night groups (Table 3).

There were significant interactions between gender and sleep duration for total WBC and neutrophil counts (both $P < 0.001$), and for the ratio T-helper naive/memory cell counts ($CD4^+RA^+/CD4^+RO^+$), which was associated with sleep duration only in boys ($r = 0.110$, $P = 0.041$).

3.3. Markers of immune function, inflammation and stress in relation to sleep duration

Significant negative associations between cortisol levels and sleep duration were found in the entire adolescent population ($r = –0.093$, $P = 0.007$), as well as in girls alone ($r = –0.114$, $P = 0.015$). Similar negative trends were found when comparing cortisol levels between the three sleep groups (Table 4, Fig. 1), but they did not reach statistical significance.

With regard to cytokine levels in the different sleep groups, inverted-U trends were observed for all variables analyzed, with the highest values in the 8–8.99 h/night group (Table 4, Fig. 1). These differences were significant for IL-4 levels ($P = 0.002$). In contrast, the pro-inflammatory/anti-inflammatory ratio and the Th1/Th2

Table 3
Counts (cell/μL) of selected lymphocyte subsets in relation to sleep duration in European adolescents.

Lymphocyte subsets	All			Boys			Girls		
	<8 h ($n = 227$)	8–8.99 h ($n = 307$)	≥9 h ($n = 203$)	<8 h ($n = 109$)	8–8.99 h ($n = 137$)	≥9 h ($n = 108$)	<8 h ($n = 118$)	8–8.99 h ($n = 170$)	≥9 h ($n = 95$)
$CD3^+$ (T-cells)	1539 ± 389*	1458 ± 382*	1481 ± 367*	1550 ± 387*	1457 ± 428*	1482 ± 338*	1528 ± 392	1458 ± 343	1481 ± 398
$CD3^+CD45RA^+$	907 ± 290	873 ± 269	897 ± 266	946 ± 305	886 ± 282	912 ± 252	870 ± 272	862 ± 259	879 ± 281
$CD3^+CD45RO^+$	618 ± 199*	576 ± 193*	567 ± 169*	585 ± 181*	558 ± 205*	553 ± 163*	648 ± 211	590 ± 182	583 ± 174
$CD4^+$ (T-helper)	869 ± 233*	805 ± 215*	827 ± 226*	876 ± 234*	801 ± 223*	814 ± 214*	864 ± 232	808 ± 209	841 ± 240
$CD4^+CD45RA^+$	485 ± 179	466 ± 178	482 ± 173	501 ± 194	477 ± 186	484 ± 171	470 ± 164	457 ± 171	479 ± 176
$CD4^+CD45RO^+$	383 ± 123*	337 ± 105*	338 ± 99*	371 ± 116*	321 ± 93*	322 ± 99*	394 ± 128	350 ± 112	355 ± 97*
$CD8^+$ (T-cytotoxic)	590 ± 200	560 ± 196	567 ± 177	587 ± 195	558 ± 220	571 ± 176	593 ± 204	562 ± 175	562 ± 179
$CD8^+CD45RA^+$	404 ± 154	383 ± 141	394 ± 134	421 ± 164	382 ± 151	393 ± 129	389 ± 143	383 ± 133	395 ± 141
$CD8^+CD45RO^+$	179 ± 97	177 ± 99	168 ± 77	160 ± 73	177 ± 114	169 ± 79	196 ± 111	177 ± 86	167 ± 74
$CD3^-CD16^+56^+$ (NK)	371 ± 160	324 ± 151	329 ± 171	326 ± 162	340 ± 156	328 ± 180	308 ± 158	312 ± 147	329 ± 161
$CD3^-CD19^+$ (B-cells)	272 ± 106	270 ± 112	272 ± 102	288 ± 115	293 ± 122	283 ± 110	257 ± 95	252 ± 99	259 ± 90
$CD4^+/CD8^+$	1.56 ± 0.44	1.57 ± 0.65	1.53 ± 0.43	1.59 ± 0.49	1.64 ± 0.82	1.51 ± 0.46	1.53 ± 0.39	1.52 ± 0.46	1.56 ± 0.38
$CD3^+RA^+/CD3^+RO^+$	1.59 ± 0.63	1.63 ± 0.63	1.67 ± 0.59	1.74 ± 0.66	1.71 ± 0.66	1.75 ± 0.62	1.44 ± 0.56	1.57 ± 0.60	1.58 ± 0.56
$CD4^+RA^+/CD4^+RO^+$	1.39 ± 0.68	1.49 ± 0.69	1.52 ± 0.68	1.47 ± 0.70	1.59 ± 0.74	1.63 ± 0.80	1.31 ± 0.66	1.41 ± 0.63	1.39 ± 0.49
$CD8^+RA^+/CD8^+RO^+$	2.79 ± 1.72	2.68 ± 1.47	2.75 ± 1.45	3.19 ± 1.95	2.76 ± 1.49	2.73 ± 1.37	2.42 ± 1.40	2.61 ± 1.46	2.79 ± 1.55

Data presented as mean ± SD.
Lymphocyte populations are designated by their cell membrane markers.
$CD45RA^+$, naive cells; $CD45RO^+$, memory cells; NK, natural killer cells.
* Significant differences between sleep categories as assessed by analysis of covariance ($P < 0.05$), adjusting for gender (when analyzing the entire population), center, Tanner stage, and standardized body mass index.

Table 4
Serum levels of immune and inflammatory proteins in relation to sleep duration in European male and female adolescents.

Immune and inflammatory proteins	All			Boys			Girls		
	<8 h (n = 274)[a]	8–8.99 h (n = 358)[b]	≥9 h (n = 237)[c]	<8 h (n = 113)[d]	8–8.99 h (n = 160)[e]	≥9 h (n = 127)[f]	<8 h (n = 161)[g]	8–8.99 h (n = 198)[h]	≥9 h (n = 110)[i]
Cortisol (μg/dL)	13.4 ± 7.5	12.7 ± 6.5	11.5 ± 5.2	12.6 ± 5.4	12.2 ± 5.1	11.0 ± 4.3	14.0 ± 8.7	13.0 ± 7.3	12.0 ± 6.1
CRP (mg/L)	1.07 ± 1.97	1.19 ± 4.87	0.99 ± 2.61	1.21 ± 2.57	1.67 ± 7.15	1.21 ± 3.41	0.98 ± 1.40	0.80 ± 1.23	0.74 ± 1.11
IL-1 (pg/mL)	1.02 ± 3.00	1.21 ± 3.44	0.73 ± 1.41	1.29 ± 4.06	1.36 ± 4.36	0.76 ± 1.42	0.82 ± 1.83	1.07 ± 2.44	0.70 ± 1.40
IL-2 (pg/mL)	6.17 ± 15.15	8.52 ± 22.32	5.81 ± 9.49	7.84 ± 20.85	9.61 ± 28.72	6.11 ± 9.61	4.91 ± 8.65	7.60 ± 15.06	5.48 ± 9.40
IL-4 (pg/mL)	177.0 ± 340.4*	200.9 ± 353.3*	125.0 ± 233.3*	188.6 ± 346.1	255.8 ± 402.8	167.2 ± 272.4	168.4 ± 337.0*	155.3 ± 300.0*	77.76 ± 168.7*
IL-5 (pg/mL)	1.80 ± 2.78	2.56 ± 4.94	1.73 ± 2.72	1.74 ± 2.23	2.83 ± 5.01	2.32 ± 3.35	1.83 ± 3.13*	2.33 ± 4.90*	1.07 ± 1.54*
IL-6 (pg/mL)	19.9 ± 25.5	23.6 ± 30.3	20.3 ± 31.4	21.3 ± 26.3	26.8 ± 34.2	27.6 ± 38.9	18.8 ± 24.9*	20.9 ± 26.4*	12.1 ± 16.5*
IL-10 (pg/mL)	31.2 ± 149.1	37.2 ± 126.9	25.2 ± 71.2	43.0 ± 222.5	34.2 ± 108.3	24.6 ± 53.6	22.4 ± 43.6	39.7 ± 141.0	26.0 ± 87.1
TNF-α (pg/mL)	5.90 ± 3.36	6.78 ± 6.45	6.06 ± 2.99	6.76 ± 3.68	7.95 ± 8.62	6.58 ± 3.29	5.26 ± 2.30	5.80 ± 3.49	5.47 ± 2.50
IFN-γ (pg/mL)	9.0 ± 30.3	15.6 ± 72.3	8.8 ± 28.1	9.4 ± 38.1	20.3 ± 99.7	8.7 ± 33.3	8.7 ± 23.0	11.6 ± 35.4	9.0 ± 21.1
Pro-/Anti	0.23 ± 0.30*	0.19 ± 0.25*	0.27 ± 0.29*	0.28 ± 0.37	0.20 ± 0.29	0.24 ± 0.30	0.19 ± 0.24*	0.19 ± 0.22*	0.29 ± 0.27*
Th1/Th2	0.68 ± 0.90*	0.63 ± 0.85*	1.00 ± 1.64*	0.75 ± 0.93	0.56 ± 0.81	0.83 ± 1.28	0.62 ± 0.87*	0.69 ± 0.88*	1.20 ± 1.95*

N indicates the maximum number of cases with valid data; valid cases for cytokine ratios were: [a] 238, [b] 290, [c] 205, [d] 101, [e] 131, [f] 109, [g] 137, [h] 158, [i] 96.
Data are presented as mean ± SD.
CRP, C-reactive protein; IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, γ-interferon; Pro-/Anti, pro-inflammatory/anti-inflammatory cytokine ratio = (TNF-α + IL-1)/(IL-4 + IL-10); Th-1/Th-2 cytokine ratio = (TNF-α + IFN-γ + IL-2)/(IL-4 + IL-5 + IL-6).
* Significant differences between sleep categories as analyzed by analysis of covariance ($P < 0.05$), adjusting for gender (when analyzing the entire population), center, Tanner stage, and standardized body mass index.

cytokine ratio revealed U-shaped trends in relation to sleep, with the 8–8.99 h/night group presenting the lowest values ($P = 0.008$ and $P = 0.009$, respectively) (Table 4, Fig. 1).

Significant gender interactions were found in the associations between interleukin levels and sleep duration. In boys, longer sleep was correlated with higher IL-10 levels ($r = 0.142$, $P = 0.009$). In girls, significant differences between sleep groups were observed, with the ≥9 h/night group showing the lowest values of IL-4 ($P = 0.001$), IL-5 ($P = 0.013$), and IL-6 ($P = 0.024$), and the highest pro-inflammatory/anti-inflammatory and Th1/Th2 cytokine ratios (Table 4); in girls, these ratios did not show a U-trend but rather a linear association with sleep duration ($r = 0.104$ for the pro-inflammatory/anti-inflammatory ratio, and $r = 0.128$ for the Th1/Th2 ratio, both $P < 0.05$).

4. Discussion

The study revealed significant associations between self-reported habitual sleep duration and immune cell counts, notably, total WBC, neutrophils, T-helper and memory cells, as well as between sleep duration and circulating cytokine profiles in this European adolescent population.

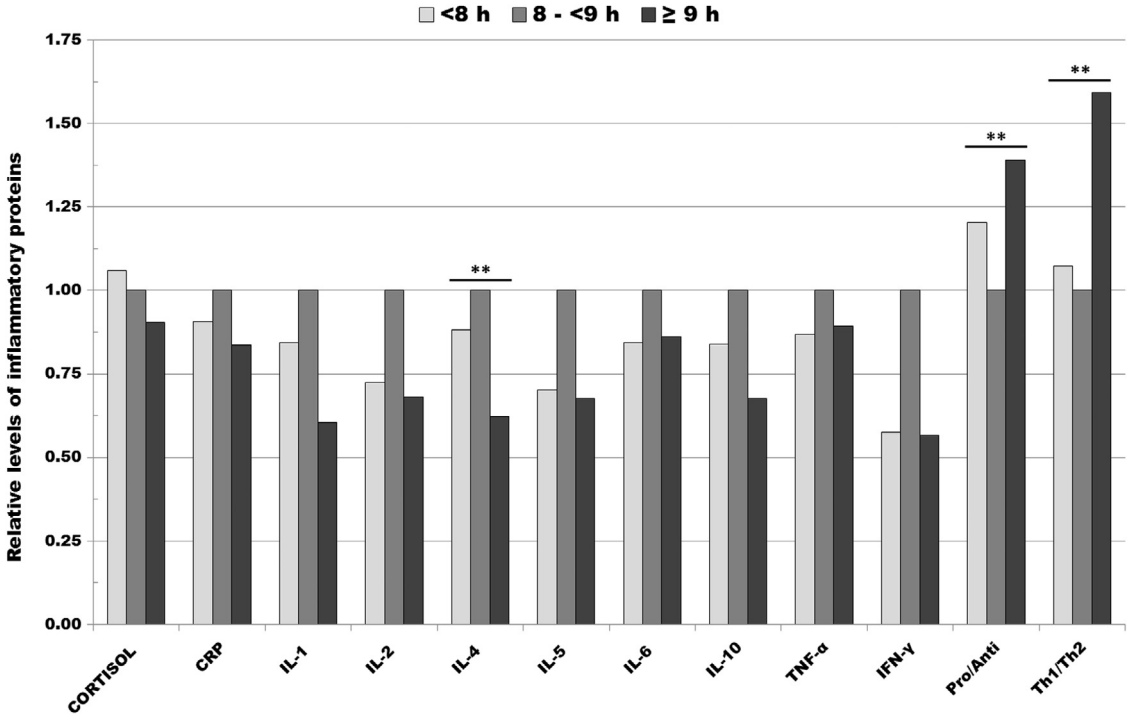

Fig. 1. Relative serum levels of cortisol, C-reactive protein (CRP), interleukins (IL)-1, -2, -4, -5, -6 and -10, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), pro-inflammatory/anti-inflammatory cytokine ratio [Pro/Anti; calculated as (TNF-α + IL-1)/(IL-4 + IL-10) and Th1/Th2 cytokine ratio [calculated as (TNFα + IFN-γ + IL-2)/(IL-4 + IL-5 + IL-6)]. For simplicity of representation, the average value in the 8–8.99 h/night sleep group has been considered the reference unit for each variable. Asterisks indicate significant differences between sleep categories according to analysis of covariance, adjusting for gender, center, Tanner stage, and standardized body mass index (*$P < 0.05$, **$P < 0.01$).

Although analyzing sleep habits in our adolescents was not the aim of the present study, it is worth mentioning that no differences were found in average sleep duration between weekdays and weekends, which is surprising considering the current literature on sleep habits in adolescents, which reports compensatory sleep during the weekends [26–28]. Our data on sleep were self-reported by participants, similar to most of the other studies available, and the questionnaire used was validated during a pilot study. It is important to note, however, that self-reported questionnaires may provide accurate estimations of total sleep time during school nights, but more caution would be required when handling data on weekend total sleep time [26].

With regard to the relationship between sleep duration and immune cell counts, we found that total WBC, neutrophil and monocyte counts increased with shorter sleep duration. This is in agreement with previous studies in adults that associated lack of sleep with higher WBC counts, mainly neutrophils and/or monocytes [12–15,29], and with the notion of wakefulness being linked with enhanced innate immunity [5,7]. We also observed that adolescents reporting shorter sleep had higher counts of total T- and memory T-cells ($CD3^+$ and $CD3^+RO^+$), due to increased T-helper cell counts ($CD4^+$ and $CD4^+RO^+$). Interpretation of these findings is less straightforward when the available literature is taken into account; however, the consistency observed in the total sample and in boys and girls separately suggests an association worth considering. Sleep is thought to benefit the interaction between lymphocytes and antigen-presenting cells and, in consequence, the formation and activation of memory cells [6,7]. Therefore, higher circulating T-cell counts would be expected with longer sleep, the opposite of what we have found. On the other hand, cortisol has been shown to redistribute T-cells between the circulation and the immune organs, so that when cortisol levels rise, T-cell counts decrease [6,7]. In our study population, however, both cortisol levels and circulating T-cell counts were negatively associated with sleep duration, suggesting that there must be another explanation for this increase in T-cell counts with short sleep duration in the adolescents. It could be questioned whether this higher memory cell count indicates greater exposure to immune insults. Available data on the relationships between immune cell counts and sleep corresponds mostly to experiments of controlled, short-term sleep deprivation, which have shown either no effect on circulating T-cell counts [30,31], at least in the morning hours, or a decrease in circulating T-cell counts [12]. In addition, the few studies that, like ours, focused on self-reported habitual sleep did not find clear associations between T-cell counts and sleep duration [32], highlighting the need for further research on this topic.

Sleep plays a key role in the production of cytokines; night time and/or sleep have been associated with maximum levels of pro-inflammatory cytokines [6]. Inflammation associated with sleep can be beneficial, as it helps fight external or internal threats with fewer undesirable secondary symptoms than when it takes place during the day or wakefulness [7]. In the present study, adolescents reporting 8–8.99 h of sleep per night presented a tendency for higher serum levels of interleukins, TNF-α, and IFN-γ than those sleeping <8 h/night and ≥9 h/night; however, when considering the ratio between pro-inflammatory (TNFα and IL-1) and anti-inflammatory (IL-4 and IL-10) cytokines, the 8–8.99 h/night group showed the lowest values. The higher pro-/anti-inflammatory ratio observed in the ≥9 h/night group would be coherent with the pro-inflammatory nature of sleep. As for the higher values observed in the <8 h/night group compared with the 8–8.99 h/group, experiments on controlled, prolonged sleep restriction conditions have shown increased circulating levels of pro-inflammatory proteins, such as IL-6, IL-1β, or CRP [30,33]. It seems that the initial reduction of circulating inflammatory markers reported by acute sleep deprivation studies would be counteracted when sleep restriction is sustained. Further research is needed to properly elucidate the effects of prolonged and/or habitual sleep restriction on inflammatory cytokines, especially in pediatric populations.

We also observed increased Th1/Th2 cytokine ratios in adolescents who reported sleeping ≥9 h/night. Sleep has been found to promote a switch toward Th1-type cytokine production that would contribute to enhanced cell-mediated immunity [6,7,34]. Other authors, on the contrary, have proposed the same effect for lack of sleep [8]. However, experiments on controlled sleep restriction, both acute and prolonged, seem to agree on finding a shift toward enhanced Th2-type activity as a consequence of lack of sleep [31,34,35]. In our study, there was mainly a shift towards Th1-type cytokines in adolescents sleeping ≥9 h/night. One reason for the apparent discrepancy in some of the literature consulted could be the variability in the cytokines considered when calculating the Th1/Th2 ratio (e.g. IL-12/IL-10, or IFN-γ/IL-4 ratios, etc.), but in any case further studies will be needed to clarify the effect of habitual sleep duration on cytokine profile. Despite this consideration, the fact that the group sleeping 8–8.99 h/night showed the lowest pro-/anti-inflammatory and Th1/Th2-type ratios leads us to suggest a range between 8 and 9 h/night as the optimal sleep duration in adolescents, at least with respect to immune function.

Finally, it is worth noting that the findings in boys and girls differed in our study. The associations between cell counts and sleep duration were found mainly in boys, whereas the associations with cytokine levels and ratios were found mainly in girls. Our results point at gender-specific effects of lack of sleep, consistent with other authors' findings [36]. This gender interaction can be related to sex hormones, as they modulate immune function at several levels [37–39]. In this sense, it is also important to keep in mind that no information was available regarding the menstrual phase of the girls, which might have introduced a larger variation into this group, making it more difficult to elucidate the influence of sleep duration.

The current study has limitations and strengths that should be addressed. The first limitation is that the cross-sectional design does not enable the inference of causality. Also, due to the large sample and the broader objectives of the HELENA-CSS, blood samples were obtained only at a single point in the day, which prevents us from considering the circadian variations in the values of the immune parameters studied – information that would have provided a more accurate picture of the relationships between sleep and the immune system. On the one hand, the samples taken represented a snapshot of the immune system in the morning of the sampling; this can be influenced by the participants' actual sleep duration on the previous night, which might or might not have coincided with their usual sleep duration, and by their morning- or evening-type profiles. On the other hand, although blood extractions were performed at 08:00, the number of samples to be taken on the same day could sometimes extend the sampling period; therefore, we cannot rule out that the values of some parameters had experienced some degree of circadian variation. However, the error accounted for by these limitations is likely to be minimized precisely by the large sample size studied, which is one of the main strengths of the present study. Our study is one of the very few to examine the associations between habitual sleep duration and immune status, and is the first to be conducted on adolescents. As far as we are aware, data in relation to sleep and immune parameters in children and adolescents are available from studies on diseases, but no information has been published on the relationship between immune status and sleep duration in healthy adolescents. The findings from the present work may be useful to establish the bases for more specific studies.

In conclusion, self-reported sleep duration in this European adolescent population was inversely associated with cortisol levels and circulating WBC, neutrophil, monocyte, total T-, T-helper and memory T-lymphocyte counts. Adolescents reporting 8–8.99 h of sleep per night

showed higher average values of serum cytokines, but a lower pro-inflammatory profile. Therefore, we suggest 8–8.99 h/night as the optimal sleep duration in adolescents concerning immune function.

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Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: http://dx.doi.org/10.1016/j.sleep.2014.04.010.

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Appendix. HELENA Study Group

Co-ordinator: Luis A. Moreno.

Core Group members: Luis A. Moreno, Fréderic Gottrand, Stefaan De Henauw, Marcela González-Gross, Chantal Gilbert.

Steering Committee: Anthony Kafatos (President), Luis A. Moreno, Christian Libersa, Stefaan De Henauw, Sara Castelló, Fréderic Gottrand, Mathilde Kersting, Michael Sjöstrom, Dénes Molnár, Marcela González-Gross, Jean Dallongeville, Chantal Gilbert, Gunnar Hall, Lea Maes, Luca Scalfi.

Project Manager: Pilar Meléndez.

1. Universidad de Zaragoza (Spain): Luis A. Moreno, Jesús Fleta, José A. Casajús, Gerardo Rodríguez, Concepción Tomás, María I. Mesana, Germán Vicente-Rodríguez, Adoración Villarroya, Carlos M. Gil, Ignacio Ara, Juan Revenga, Carmen Lachen, Juan Fernández Alvira, Gloria Bueno, Aurora Lázaro, Olga Bueno, Juan F. León, Jesús Mª Garagorri, Manuel Bueno, Juan Pablo Rey López, Iris Iglesia, Paula Velasco, Silvia Bel, Luis A. Gracia Marco, Theodora Mouratidou.
2. Consejo Superior de Investigaciones Científicas (Spain): Ascensión Marcos, Esther Nova, Sonia Gómez-Martínez, Ligia Esperanza Díaz, Javier Romeo, Ana Veses, Belén Zapatera, Tamara Pozo, David Martínez.
3. Université de Lille 2 (France): Laurent Béghin, Christian Libersa, Frédéric Gottrand, Catalina Iliescu, Juliana Von Berlepsch.
4. Research Institute of Child Nutrition Dortmund, Rheinische Friedrich-Wilhelms-Universität Bonn (Germany): Mathilde Kersting, Wolfgang Sichert-Hellert, Ellen Koeppen.
5. Pécsi Tudományegyetem (University of Pécs) (Hungary): Dénes Molnar, Eva Erhardt, Katalin Csernus, Katalin Török, Szilvia Bokor, Enikö Nagy, Orsolya Kovács, Judit Répasi.
6. University of Crete School of Medicine (Greece): Anthony Kafatos, Caroline Codrington, María Plada, Angeliki Papadaki, Katerina Sarri, Anna Viskadourou, Christos Hatzis, Michael Kiriakakis, George Tsibinos, Constantine Vardavas, Manolis Sbokos, Eva Protoyeraki, Maria Fasoulaki.
7. Institut für Ernährungs- und Lebensmittelwissenschaften – Ernährungphysiologie, Rheinische Friedrich-Wilhelms-Universität (Germany): Peter Stehle, Klaus Pietrzik, Marcela González-Gross, Christina Breidenassel, Andre Spinneker, Jasmin Al-Tahan, Miriam Segoviano, Anke Berchtold, Christine Bierschbach, Erika Blatzheim, Adelheid Schuch, Petra Pickert.
8. University of Granada (Spain): Manuel J. Castillo, Ángel Gutiérrez, Francisco B. Ortega, Jonatan R. Ruiz, Enrique G. Artero, Vanesa España, David Jiménez-Pavón, Palma Chillón, Cristóbal Sánchez-Muñoz, Magdalena Cuenca.
9. Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (Italy): Davide Arcella, Elena Azzini, Emma Barison, Noemi Bevilacqua, Pasquale Buonocore, Giovina Catasta, Laura Censi, Donatella Ciarapica, Paola D'Acapito, Marika Ferrari, Myriam Galfo, Cinzia Le Donne, Catherine Leclercq, Giuseppe Maiani, Beatrice Mauro, Lorenza Mistura, Antonella Pasquali, Raffaela Piccinelli, Angela Polito, Raffaella Spada, Stefania Sette, Maria Zaccaria, Romana Roccaldo.
10. University of Napoli 'Federico II' Department of Food Science (Italy): Luca Scalfi, Paola Vitaglione, Concetta Montagnese.
11. Ghent University (Belgium): Ilse De Bourdeaudhuij, Stefaan De Henauw, Tineke De Vriendt, Lea Maes, Christophe Matthys, Carine Vereecken, Mieke de Maeyer, Charlene Ottevaere, Inge Huybrechts.
12. Medical University of Vienna (Austria): Kurt Widhalm, Katharina Phillipp, Sabine Dietrich, Birgit Kubelka, Marion Boriss-Riedl.
13. Harokopio University (Greece): Yannis Manios, Eva Grammatikaki, Zoi Bouloubasi, Tina Louisa Cook, Sofia Eleutheriou, Orsalia Consta, George Moschonis, Ioanna Katsaroli, George Kraniou, Stalo Papoutsou, Despoina Keke, Ioanna Petraki, Elena Bellou, Sofia Tanagra, Kostalenia Kallianoti, Dionysia Argyropoulou, Katerina Kondaki, Stamatoula Tsikrika, Christos Karaiskos.
14. Institut Pasteur de Lille (France): Jean Dallongeville, Aline Meirhaeghe.
15. Karolinska Institutet (Sweden): Michael Sjöstrom, Jonatan R. Ruiz, Francisco B. Ortega, María Hagströmer, Anita Hurtig Wennlöf, Lena Hallström, Emma Patterson, Lydia Kwak, Julia Wärnberg, Nico Rizzo.
16. Asociación de Investigación de la Industria Agroalimentaria (Spain): Jackie Sánchez-Molero, Sara Castelló, Elena Picó, Maite Navarro, Blanca Viadel, José Enrique Carreres, Gema Merino, Rosa Sanjuán, María Lorente, María José Sánchez.
17. Campden BRI (UK): Chantal Gilbert, Sarah Thomas, Elaine Allchurch, Peter Burgess.
18. SIK – Institutet foer Livsmedel och Bioteknik (Sweden): Gunnar Hall, Annika Astrom, Anna Sverkén, Agneta Broberg.
19. Meurice Recherche & Development asbl (Belgium): Annick Masson, Claire Lehoux, Pascal Brabant, Philippe Pate, Laurence Fontaine.
20. Campden and Chorleywood Food Development Institute (Hungary): Andras Sebok, Tunde Kuti, Adrienn Hegyi.
21. Productos Aditivos SA (Spain): Cristina Maldonado, Ana Llorente.
22. Cárnicas Serrano SL (Spain): Emilio García.
23. Cederroth International AB (Sweden): Holger von Fircks, Marianne Lilja Hallberg, Maria Messerer.
24. Lantmännen Food R&D (Sweden): Mats Larsson, Helena Fredriksson, Viola Adamsson, Ingmar Börjesson.
25. European Food Information Council (Belgium): Laura Fernández, Laura Smillie, Josephine Wills.

Universidad Politécnica de Madrid (Spain): Marcela González-Gross, Jara Valtueña, David Jiménez-Pavón, Ulrike Albers, Raquel

Pedrero, Gonzalo Palacios, Agustín Meléndez, Pedro J. Benito, Juan José Gómez Lorente, David Cañada, Alejandro Urzanqui, Juan Carlos Ortiz, Francisco Fuentes, Rosa María Torres, Paloma Navarro.

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